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Chronic Bioassays of Chlorinated Humic Acids in B6C3F1 Mice

by Benjamin L. Van Duuren,* Susan Melchionne,* Irving Seidman,† and Michael A. Pereira‡

Humic acids (Fluka), chlorinated to carbon:chlorine (C:Cl) ratios of 1:1 and 1:0.3, were administered to B6C3F1 mice, 50 males and 50 females per group, in the drinking water at a total organic carbon (TOC) level of 0.5 g/L. The mice were 6 to 8 weeks old at the beginning of the bioassays. The doses used were based on short-term (8 weeks) evaluations for toxicity, palatability, and weight gain. The chronic bioassays included the following control groups: unchlorinated humic acids (0.5 g/L), no-treatment (100 males and 100 females), dibromoethane (DBE, 2.0 mM in drinking water; positive control) and 0.44% sodium chloride in drinking water, i.e., at the same concentration as those receiving chlorinated humic acids. The chlorinated humic acids were prepared freshly and chemically assayed once per week. All chemicals were, with the exception of DBE, administered for 24 months; DBE was administered for 18 months. The volumes of solutions consumed were measured once weekly. All treatment groups showed normal weight gain except the DBE group. At the completion of exposure, the animals were sacrificed and necropsied, and tissue sections were taken for histopathology. No markedly significant increases in tumor incidences were evident in any of the organs and tissues examined in the chlorinated humic acid groups compared to unchlorinated humic acids and the no-treatment control groups. DBE caused the expected high incidence of squamous carcinomas of the forestomach. The chlorinated humic acids tested contained direct-acting alkylating agents, based on their reactivity with *p*-nitrobenzylpyridine (PNBP), and showed mutagenic activity in *S. typhimurium*.

Introduction

The occurrence of low molecular weight halogenated aliphatic and olefinic hydrocarbons in the environment and their potentially adverse human health effects has become the subject of intensive research during the past 15 to 20 years. The upsurge of research in this area was spurred by the demonstration of the carcinogenic activity of vinyl chloride (*1,2*) and of other low molecular weight, halogenated, saturated and unsaturated hydrocarbons (*3–6*). These studies also focused on the chlorination products of humic and fulvic acids of water as sources of these compounds. This subject has been recently reviewed (*7*). A substantial number of the compounds formed during the chlorination of water containing humic acids have been isolated, characterized, and in some instances quantitated (*8–13*). The nature and amounts of these by-products vary with the source of the humic acids, which are a poorly defined group of materials. Other factors that affect the nature of the products include pH, duration of chlorination, and extent of available analytical procedures brought to bear on the problem. The nonvolatile, high molecular weight products formed during the chlorination of humic acids have recently received some attention as well (*14*).

The purpose of the present study was to determine whether the mixture of chlorination products would show carcinogenic activity in chronic bioassays in laboratory animals when these substances were administered in drinking water. For such experiments, where high doses were required for chronic tests in animals, it was clearly infeasible to concentrate humic acids (HA) from drinking water. A commercial source of humic acid, therefore, was used. The validity of this decision was confirmed in studies (*15*) before beginning the chronic bioassay described here.

B6C3F1 mice were selected for the chronic bioassay because of the considerable background information available on these mice. They have been used extensively in the carcinogenicity testing of many chemicals, formerly under the auspices of the carcinogenesis testing program of the National Cancer Institute and in recent years as part of the National Toxicology Program. The history, growth patterns, and lifespans (*16*), as well as incidence of spontaneous tumors (*17*), in these mice have been carefully documented. B6C3F1 mice were also used recently in this laboratory for the chronic

*Laboratory of Organic Chemistry and Carcinogenesis, Institute of Environmental Medicine, New York University Medical Center, New York, NY 10016.
†Department of Pathology, New York University Medical Center, New York, NY 10016.
‡Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

bioassay of potential metabolites of the carcinogen dibromoethane administered in drinking water (*18*).

While the chronic bioassay described in this report was underway, there were developments in other laboratories concerning the mutagenicity of chlorinated humic acids (*11,14,15,19*) and their possible initiating activity in two-stage carcinogenesis in SENCAR mice (*15*).

As part of the chronic bioassay described here, it was essential to select chlorination conditions that were reasonably close to actual chlorination conditions used to disinfect water for human consumption. Also, it was necessary to decide on concentrations of chlorinated humic acids that resulted in acceptable palatability to test animals, normal weight gains, and normal or close-to-normal lifespans. In addition, chlorination conditions had to be closely scrutinized to allow for sufficient stability of products and test solutions compatible with the protocols for the chronic bioassay. The chronic bioassay was preceded by short-term (8-week) assays of these preparations.

As a part of the chronic bioassay, the alkylating activity of the chlorinated humic acids was determined by their reactivity with *p*-nitrobenzylpyridine (PNBP). This assay was also used to determine the stability of the chlorinated products during storage and use as test solutions in drinking water. In addition, the mutagenicity of the chlorinated humic acids was determined in *S. typhimurium* TA 98 and TA 100.

Methods

Animals

Male and female B6C3F1 mice (Harlan Sprague-Dawley, Indianapolis, IN) were housed, six per stainless-steel cage, on hardwood chips and fed Purina Rodent Laboratory Chow. Males and females were housed in separate animal rooms. Control groups, i.e., those receiving drinking water, sodium chloride, or unchlorinated humic acids, were housed in the same rooms as those receiving chlorinated humic acids. The dibromoethane (DBE) positive control groups were housed in well-ventilated hoods having an air flow of at least 100 linear ft/min. Animal housing areas were maintained at 22–24°C.

Subchronic Bioassay

A short-term test of 8 weeks duration was conducted at three total organic carbon (TOC) levels (0.1, 0.2, and 0.5 g/L), all three at carbon:chlorine (C:Cl) ratios of 1:1 and 1:0.3. There were five mice per group for each of these short-term tests. The three control groups, also five mice per group, received drinking water only, sodium chloride (NaCl, 4.4 g/L), or unchlorinated humic acids (TOC levels 0.1, 0.2, and 0.5 g/L). During the 6-week treatment period, body weight and solution of water intake were recorded once weekly. Treatment was discontinued at the end of 6 weeks, and all animals were sacrificed and completely necropsied at the end of 8 weeks. Tissue sections from 12 organs were taken for histopathology from one animal of each group. These organs were: tongue, esophagus, stomach, (glandular- and forestomach), duodenum, ileum, cecum, colon, rectum, urinary bladder, kidney, lung, and liver. Organs from all groups were clinically and microscopically normal. Body weight gain and fluid intake were normal for all groups except for those receiving sodium chloride in their drinking water, which showed a slight elevation in fluid intake in both sexes.

Table 1. Protocol for chronic bioassay of chlorinated humic acids in B6C3F1 mice.

Fifty females and fifty males per group.
Test duration: 24 months
Test materials at 500 mg TOC/L
Unchlorinated humic acids
Chlorinated humic acids, C:Cl, 1:1
Chlorinated humic acids, C:Cl, 1:0.3
Control groups
Sodium chloride, 4.4 g/L
No treatment[a]
Dibromoethane,[b] 2 mM = 0.375 g/L
Total mice: 700

[a] 100 animals/group.
[b] Test duration: 18 months.

Chronic Bioassay

Based on these short-term evaluations, the highest TOC level, 0.5 g/L at C:Cl ratios 1:1 and 1:0.3, was selected for the chronic bioassay. The humic and chlorinated humic acid solutions were prepared freshly once per week as described below under chemicals. The detailed protocols for these long-term bioassays are shown in Table 1. Solution and water intakes and body weights were recorded monthly. Animals were examined once per week and palpated for the presence of internal lesions or any clinical signs of toxicity. Animals in moribund condition were sacrificed by cervical dislocation. During the first 12 months of the chronic bioassay, very few animals died. Tissue samples were taken at necropsy for histopathology of the following organs: lung, stomach, liver, kidneys, urinary bladder, colon, rectum, and gonads. During the second year of the test, the number of tissues taken at necropsy was expanded to include 26 tissues and organs as used in the National Toxicology Program (*20*). One section from each of these tissues was taken from each animal at death. All samples for histopathology were fixed in formalin, blocked, cut, and stained with hematoxylin and eosin. Portions of tissues not used for histopathology were also stored in formalin in heat-sealed plastic bags for future reference if needed.

Preparation and Chemical Analysis of Humic Acid and Chlorinated Humic Acid Solutions

Only those aspects of the chemical phases of the work pertaining to bioassays are given here. Detailed studies

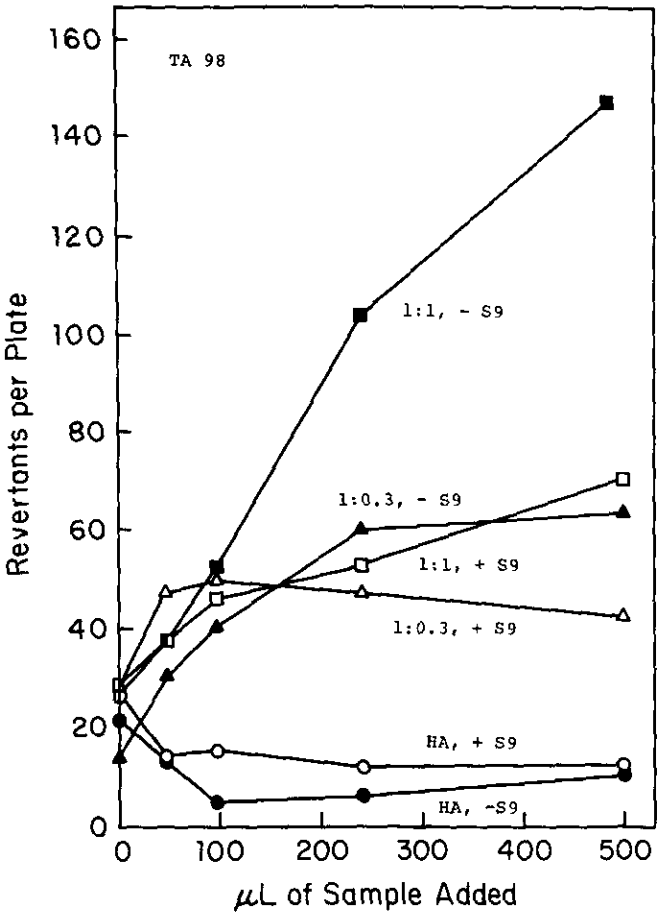

FIGURE 1. Dose response of unchlorinated humic acid (HA) and freshly prepared chlorinated humic acids at C:Cl 1:1 and 1:0.3 in *S. typhimurium*, TA 98 in the absence (–S9) and in the presence (+S9) of microsomal activating system.

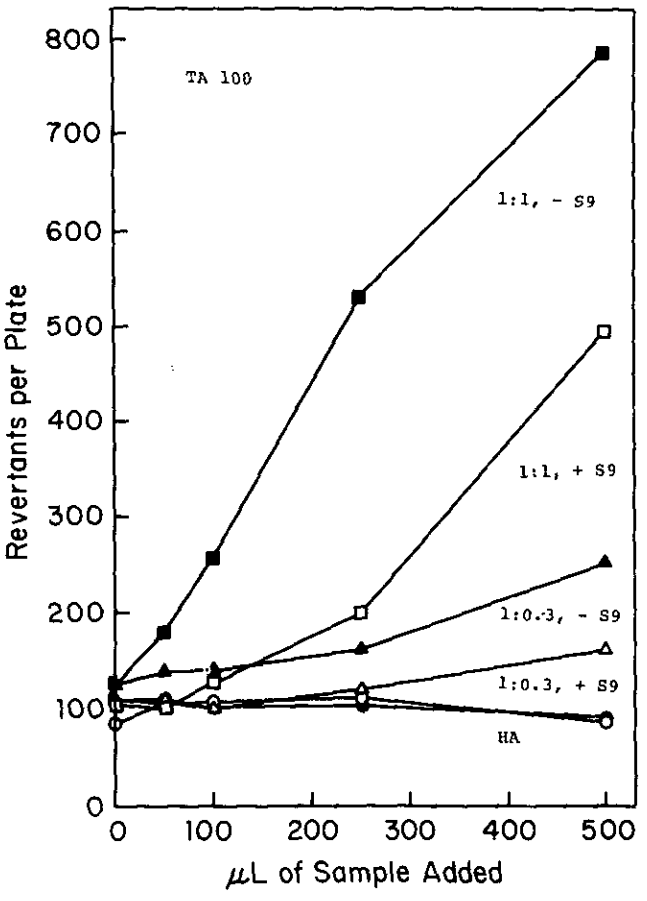

FIGURE 2. Dose response of unchlorinated humic acid (HA) and freshly prepared chlorinated humic acids at C:Cl, 1:1 and 1:0.3 in *S. typhimurium*, TA 100 in the absence (–S9) and in the presence (+S9) of microsomal activating system.

on fractionation by molecular weight and other chemical studies will be reported elsewhere.

Humic acids (Fluka, Switzerland) were used throughout this work. Stock solutions of humic acids for subsequent chlorination were prepared as follows: a solution of humic acids, 5.0 g in 400 mL of 0.02 N sodium hydroxide (NaOH), was stirred for 2 hr at room temperature and the slightly acidic solution adjusted to pH 7.0 with 0.1 N NaOH. The solution was centrifuged at 2300 g for 90 min. The clear, dark-brown supernatant solution was decanted, diluted to 1 L with distilled water, and adjusted to pH 7.0. This solution was stored overnight at 4°C and filtered through Whatman glass microfiber filters GF/D and 934-AH with particle retentions of 2.7 and 1.5 μm, respectively. Freeze-dried aliquots of this humic acid stock solution were used for various analyses including TOC analysis (TOC analyses were performed at the Health Effects Research Laboratories, U.S. Environmental Protection Agency (EPA), Cincinnati, OH by using a Dohrmann organic carbon analyzer). The TOC content of this solution was 2.0 g/L.

In a typical chlorination procedure, 250 mL of the humic acid stock solution in a 1-L amber container was diluted to 475 mL with distilled water. The chlorine content of a stock solution of sodium hypochlorite (NaOCl; 4%-6%) was determined by iodimetric analysis. The exact required volume of this stock solution was diluted with distilled water, its pH adjusted to 7.0 with 6 N hydrochloric acid (HCl), and added to the humic acid stock solution to attain C:Cl ratios of 1:1 and 1:0.3.

Table 2. Consumption of chemical solutions and survival in male mice.

Treatment	Average amount/mouse/day		Number of survivors	
	mL	mg	12 Months	24 Months
Unchlorinated humic acid (HA)	5.5	2.8 (TOC)	50	45
Chlorinated HA				
C:Cl, 1:1	5.5	2.8 (TOC)	49	42
C:Cl, 1:0.3	5.7	2.9 (TOC)	50	39
Sodium chloride	6.0	26.4	49	45
Dibromoethane	3.6	1.4	48	34[a]
No treatment[b]	5.7	—	99	78

[a] Terminated at 18 months.
[b] 100 mice, other groups, 50 mice.

Table 3. Consumption of chemical solutions and survival in female mice.

Treatment	Average amount/ mouse/day		Number of survivors	
	mL	mg	12 Months	24 Months
Unchlorinated humic acid (HA)	4.2	2.1 (TOC)	49	48
Chlorinated HA				
C:Cl, 1:1	4.4	2.2 (TOC)	50	43
C:Cl, 1:0.3	4.3	2.2 (TOC)	50	41
Sodium chloride	5.0	22.0	48	44
Dibromoethane	3.3	1.2	50	39[a]
No treatment[b]	4.4	—	99	91

[a] Terminated at 18 months.
[b] 100 mice; other groups, 50 mice.

The 1-liter container was filled with distilled water, leaving no head space, stoppered, and stored at room temperature for 6 days. After reaction, the free residual chlorine content, as determined by iodimetric titration, was less than 10 mg/L. The final pH of the chlorinated solutions was in the range of 2.2 to 3.3. All solutions for bioassay were adjusted to pH 5.0 with 1 N NaOH and stored at 4°C. The chlorinated humic acid solutions for bioassay were prepared freshly once per week and were assayed for TOC and total organic halogen (TOX). Both TOC and TOX analyses were performed by the Health Effects Research Laboratory, U.S. EPA, Cincinnati, OH. These samples were also analyzed for mutagenicity (*21*) and alkylating activity (*22*).

Reactivity of Chlorinated Humic Acids with PNBP

The chlorinated humic acids were analyzed for alkylating activity in stock solutions and in bioassay solutions using the PNBP method, a widely used, but still semiquantitative procedure (*22*). It depends on the development of an unstable, colored reaction mixture after reaction of alkylating agents with PNBP and addition of base. A known alkylating agent, bromoacetic acid ($BrCH_2COOH$), was used as standard. This compound does not absorb light in the ultraviolet-visible region and is very soluble in water, the medium of choice for the present work. A standard curve was constructed by using 5.0 to 50.0 mg/L of $BrCH_2COOH$ in the presence of an excess of PNBP. The blue color was developed by adding triethylamine, and absorbance at 570 nm was measured. The same procedure was applied to aliquots of the chlorinated humic acids from various chlorination conditions, C:Cl ratios from 1:0.3 to 1:1.2, before and after freeze-drying, at various pH levels and duration of storage. All solutions assayed, including the reaction blanks $BrCH_2COOH$, PNBP, and unchlorinated and chlorinated humic acids, were run in triplicate.

Table 4. Average body weights for male and female mice.

Treatment	Average body weight, g		Range, g	
	Males	Females	Males	Females
Unchlorinated humic acid (HA)	34	27	21–38	16–32
Chlorinated HA				
C:Cl, 1:1	33	28	19–36	17–32
C:Cl, 1:0.3	34	27	20–37	17–33
Sodium chloride	35	28	22–39	16–33
Dibromoethane	28	23	15–31	14–26
No treatment	35	27	20–38	16–32

Table 5. Tumor incidences with chlorinated humic acids (TOC, 500 mg/L; C:Cl 1:1).

Site	Number	Type
Females (49 necropsied)		
Skin	2	Hemangioma
	10	Sarcoma
Lung	5	Papillary tumor
Lymph node	5	Hemangioma ($p < 0.005$)
Spleen	2	Hemangioma
Blood vessel	1	Hemangioma
Liver	3	Hemangioma
	1	Hyperplastic nodule
Stomach	6	Papilloma (forestomach)
	1	Squamous carcinoma (forestomach)
Perianal gland	1	Adenoma
Uterus/uterine horns	1	Leiomyoma
Ovaries/oviducts	3	Hemangioma ($p \cong 0.05$)
Mammary gland	2	Mammary tumor
	2	Carcinoma
Harderian/lacrimal gland	5	Papillary adenoma ($p \cong 0.05$)
Mice with tumor	42[a]	
Mice with leukemia	35	
Males (49 necropsied)		
Skin	1	Papilloma
	15	Sarcoma
Lung	16	Papillary tumor
Lymph node	2	Hemangioma
Liver	5	Hemangioma
	11	Hyperplastic nodule
	2	Hepatoma
Stomach	1	Papilloma (forestomach)
Duodenum	1	Adenocarcinoma
Anus	1	Adenoma/low-grade adenocarcinoma
Preputial gland	1	Adenoma
Harderian/lacrimal gland	4	Papillary adenoma
Mice with tumor	44[b]	
Mice with leukemia	29	

[a] 30 with solid tumor.
[b] 34 with solid tumor.

Mutagenicity of Chlorinated Humic Acids using *S. typhimurium*

Four tester strains, TA 98, TA 100, TA 1537 and TA 1538 were used in exploratory studies in the presence and absence of an S-9 liver microsomal activating system (*21*). TA 1537 and TA 1538 were less sensitive than TA 98 and TA 100 and hence were not used in further work. All bacterial toxicity and mutagenicity assays described in this report were carried out by Litton Bionetics, Inc. (Kensington, MD). Unchlorinated and

Table 6. Tumor incidences with chlorinated humic acids (TOC, 500 mg/L; C:Cl, 1:0.3).

Site	Number	Type
Females (50 necropsied)		
Skin	6	Sarcoma
Lung	4	Papillary tumor
Lymph node	2	Hemangioma ($p \cong 0.05$)
Spleen	1	Hemangioma
Liver	1	Hepatoma
	1	Hemangioma
	2	Hyperplastic nodule
Stomach	3	Papilloma (forestomach)
Duodenum	1	Adenoma
Adrenal gland	1	Undifferentiated malignant tumor
Uterus/uterine horns	1	Papillary adenoma
	2	Hemangioma
Mammary gland	2	Mammary tumor
	2	Carcinoma
Brain	2	Hemangioma ($p < 0.05$)
Harderian/lacrimal gland	3	Papillary adenoma
Ear duct gland	1	Carcinoma
Skeletal system	1	Osteoma
Mice with tumor	44[a]	
Mice with leukemia	32	
Males (50 necropsied)		
Skin	1	Papilloma
	1	Squamous carcinoma
	2	Fibroma
	15	Sarcoma
Lung	17	Papillary tumor
Lymph node	2	Hemangioma
Liver	3	Hemangioma
	10	Hyperplastic nodule
	3	Hepatoma
Stomach	1	Papilloma (forestomach)
	1	Adenocarcinoma (glandular stomach)
Testes	1	Leydig cell tumor
Preputial gland	1	Adenoma
Prostate gland	2	Adenocarcinoma ($p < 0.05$)
Harderian/lacrimal gland	2	Papillary adenoma
Skeletal system	2	Osteoma ($p < 0.05$)
Mice with tumor	43[b]	
Mice with leukemia	17	

[a] 26 with solid tumor.
[b] 36 with solid tumor.

Table 7. Tumor incidences with unchlorinated humic acids (TOC, 500 mg/L).

Site	Number	Type
Females (48 necropsied)		
Skin	1	Papilloma
	9	Sarcoma
Lung	7	Papillary tumor
Lymph node	2	Hemangioma ($p \cong 0.05$)
Spleen	1	Hemangioma
Liver	4	Hemangioma
	2	Hyperplastic nodule
Stomach	1	Papilloma (forestomach)
	1	Squamous carcinoma (forestomach)
Duodenum	1	Adenoma
	1	Adenocarcinoma
Jejunum/ileum	1	Poorly differentiated carcinoma
Thyroid gland	1	Adenoma
Mammary gland	3	Mammary tumor
Mice with tumor	44[a]	
Mice with leukemia	41	
Males (50 necropsied)		
Skin	19	Sarcoma
	1	Spindle cell tumor (benign)
Lung	17	Papillary tumor
Spleen	2	Hemangioma
Lymph node	3	Hemangioma
Liver	3	Hemangioma
	3	Hyperplastic nodule
	7	Hepatoma
Stomach	1	Papilloma (forestomach)
	1	Adenoma (glandular stomach)
	1	Adenocarcinoma (glandular stomach)
	1	Neuroendocrine tumor (glandular stomach)
Duodenum	1	Adenoma
	1	Focal atypical hyperplasia/ carcinoma *in situ*
Jejunum/ileum	1	Adenocarcinoma
Kidney	1	Cortical adenoma
Testes	1	Interstitial cell tumor
Mammary gland	1	Adenocarcinoma
Harderian/lacrimal gland	4	Papillary adenoma
Mice with tumor	42[b]	
Mice with leukemia	16	

[a] 26 with solid tumor.
[b] 39 with solid tumor.

chlorinated humic acids (C:Cl, 1:1 and 1:0.3) were tested as fresh solutions and as freeze-dried and reconstituted solutions in distilled water at three times their original concentrations. Four dose levels, two plates per dose, with and without S9 activation were assayed. Sodium azide (for TA 100) and 2-nitrofluorene (for TA 98) without S9 activation were used as positive controls; 2-aminoanthracene was used as the positive control for both tester strains in the presence of S9 (*19*).

Results

Total Organic Halogen Content

TOX analyses were performed on six to eight samples for each of the 2 chlorinated humic acids. At C:Cl 1:1 and 1:0.3, the average TOX analyses were 222 and 69.3 mg/L, respectively.

Alkylating Activity and Mutagenicity

Humic acids chlorinated at C:Cl 1:0.3 and unchlorinated starting material did not show any activity in the PNBP assay. Chlorinated humic acids at C:Cl 1:1 showed notable absorption in this assay at 570 nm. For all three samples the TOC concentration was 1.0 g/L; chlorinated humic acids, C:Cl 1:1, showed alkylating activity of > 80 ppm (equivalence of $BrCH_2COOH$). The effects of freeze-drying, pH, and storage on the alkylating activity of chlorinated humic acids are described elsewhere (*23*).

The results of the mutagenicity assays using the tester strains TA 98 and TA 100 are shown in Figures 1 and 2. The positive controls (not shown) gave the

Table 8. Tumor incidences in NaCl (4.4 g/L) control groups.

Site	Number	Type
Females (47 necropsied)		
Skin	1	Papilloma
	9	Sarcoma
Lung	11	Papillary tumor
Lymph node	1	Hemangioma
Spleen	1	Hemangioma
Liver	1	Hyperplastic nodule
Stomach	2	Papilloma (forestomach)
	1	Papilloma with focal carcinoma (forestomach)
Jejunum/ileum	1	Sarcoma
Uterus/uterine horns	2	Leiomyoma
	1	Hemangioma
	2	Sarcoma
Ovaries/oviducts	1	Hilar cell adenoma
	1	Benign cystic tumor
Vagina	1	Squamous carcinoma
Mammary gland	3	Mammary tumor
	1	Hemangioma
Harderian/lacrimal gland	3	Papillary adenoma
Adipose tissue	1	Hemangioma
Mice with tumor	40[a]	
Mice with leukemia	30	
Males (50 necropsied)		
Skin	9	Sarcoma
Lung	16	Papillary tumor
Spleen	1	Hemangioma
Lymph node	1	Hemangioma
Liver	4	Hemangioma
	11	Hyperplastic nodule
	6	Hepatoma
Stomach	1	Papilloma (forestomach)
Harderian/lacrimal gland	3	Papillary adenoma
Mice with tumor	43[b]	
Mice with leukemia	20	

[a] 27 with solid tumor.
[b] 35 with solid tumor.

Table 9. Tumor incidences in untreated control groups (100 females and 100 males).

Site	Number	Type
Females (96 necropsied)		
Skin	1	Papilloma
	19	Sarcoma
	1	Hemangioma
Lung	13	Papillary tumor
Spleen	3	Hemangioma
Liver	2	Hepatoma
	3	Hemangioma
	2	Hyperplastic nodule
Stomach	9	Papilloma (forestomach)
	1	Adenoma (glandular stomach)
Caecum	1	Leiomyoma
Thyroid gland	1	Papillary carcinoma
Uterus/uterine horns	4	Hemangioma
Ovaries/oviducts	1	Papilloma
	1	Leiomyoma
	1	Hemangioma
Clitoral gland	1	Adenoma
Mammary gland	4	Mammary tumor
	2	Carcinoma
Harderian/lacrimal gland	3	Papillary adenoma
Mice with tumor	84[a]	
Mice with leukemia	63	
Males (99 necropsied)		
Skin	1	Papilloma
	1	Squamous carcinoma
	26	Sarcoma
Lung	22	Papillary tumor
Spleen	4	Hemangioma
Lymph node	4	Hemangioma
Heart	1	Sarcoma
Blood vessels	1	Hemangioma
Liver	3	Hemangioma
	15	Hyperplastic nodule
	12	Hepatoma
Stomach	5	Papilloma (forestomach)
	2	Squamous carcinoma (forestomach)
Jejunum/ileum	1	Atypical hyperplasia (early carcinoma)
Preputial gland	1	Adenoma
	1	Hemangiosarcoma
Mammary gland	1	Mammary tumor
Harderian/lacrimal gland	4	Papillary adenoma
Mice with tumor	76[b]	
Mice with leukemia	23	

[a] 57 with solid tumor.
[b] 67 with solid tumor.

expected results (*19*). Both chlorinated humic acids (C:Cl, 1:1 and 1:0.3) showed highest activity in the absence of the S9 activating system and showed a clear dose-response pattern under the experimental conditions used. The unchlorinated material did not show significant activity compared to controls, and the revertants per plate were markedly higher at C:Cl 1:1, compared to C:Cl 1:0.3. At both C:Cl ratios, the freshly chlorinated preparations showed more than four times the activity in both tester strains in the absence of S9, compared to freeze-dried material tested under the same conditions (*23*). The mutagenic activity in both tester strains for both ratios of chlorinated humic acids 4 days after preparation and use on animal cages ranged from 52 to 69% compared to freshly prepared solutions in the absence of the S9 system.

Chronic Bioassay

The consumption of chemical solutions and survival in male and female mice in the chronic bioassays are shown in Tables 2 and 3, respectively. In both sexes, the consumption of solutions for unchlorinated and chlorinated humic acids was the same as that in untreated control groups; intake of DBE solution was depressed and of NaCl solution increased. Survival for test groups, except DBE, was the same as that for control groups.

The average body weights and body weight ranges for both sexes are shown in Table 4. The animals exposed to chemicals in the drinking water, except for the DBE group, gave the same body weight versus days-on-test curves, and, hence, the results are shown only in summary form in Table 4.

The complete pathologic findings concerning benign and malignant tumors for all test and control animals are tabulated in Table 5 through 10. The significance

Table 10. Tumor incidences in dibromoethane (2 mM) positive control group.

Site	Number	Type
Females (49 necropsied)		
Skin	1	Malignant tumor
Lung	2	Papillary tumor
Thymus	1	Malignant tumor
Spleen	1	Hemangioma
Tongue	1	Squamous carcinoma *in situ*
Esophagus	4	Papilloma
Liver	1	Hyperplastic nodule
Stomach	29	Papilloma (forestomach) ($p < 0.0005$)
	20	Squamous carcinoma (forestomach) ($p < 0.0005$)
Mammary gland	1	Mammary tumor
	1	Carcinoma
Harderian/lacrimal gland	2	Papillary adenoma
Mice with tumors	49[a]	
Mice with leukemia	5	
Males (48 necropsied)		
Esophagus	4	Papilloma ($p < 0.01$)
	4	Squamous carcinoma ($p < 0.01$)
Liver	1	Hyperplastic nodule
	1	Hepatoma
Stomach	6	Papilloma (forestomach)
	41	Squamous carcinoma (forestomach) ($p < 0.0005$)
Caecum	1	Carcinoma
Mice with tumor	48[b]	
Mice with leukemia	1	

[a] 49 with solid tumor.
[b] 48 with solid tumor.

values given in these tables were calculated by the chi-square method used in our earlier work (*18*). Metastatic tumors that occurred are not listed in Tables 5–10.

Discussion

An examination of the literature on the products formed during the chlorination of water and of humic and fulvic acids reveals that only a few of them have been examined for possible deleterious health effects in humans. Undue emphasis has been placed on halogenated methanes, particularly chloroform, because of its carcinogenic activity in rodents. High molecular weight and nonvolatile chlorinated humic acids constitute the major part of the chlorination products of humic acids, yet their chemical structures are unknown and their biological properties have only recently received attention (*14*). Other chlorination products of drinking water sources and of humic acids (*11–13*) include chlorocarboxylic acids, chlorophenols, chloroketones, and chloroacetonitriles. The potential mutagenicity and/or carcinogenicity of a few of these components have received attention (*6*).

Based on the above information, we decided to perform chronic animal bioassays, not on individual components of drinking water, but rather on the mixture of products itself, since this is what is consumed daily by humans. Such a mixture of products undoubtedly contains not only small amounts of carcinogens, but also cocarcinogens and, not to be disregarded, tumor-inhibitory agents. The importance of cocarcinogens (*24,25*) and of tumor-inhibitory agents (*26*) in environmental chemical carcinogenesis has been reviewed.

Although concentrates of humic acids and their chlorination products can conceivably be prepared directly from drinking water sources, this approach is undesirable for chronic bioassays in laboratory animals for several reasons. It is impractical to prepare these materials in sufficiently high concentrations for bioassays at maximum tolerated doses. Equally important is that, in spite of all attempts to avoid loss and degradation of some products of chlorination, e.g., volatile or highly reactive chemicals, these processes undoubtedly will occur during concentration. Impurities in organic solvents used for extraction of chlorination products from drinking water and incomplete extraction involved in these procedures only serve to compound the difficulties encountered in this approach to developing a source of concentrated mixtures of chlorinated humic acid products for chronic animal bioassays.

Fluka humic acid as well as humic/fulvic acid preparations from water sources have been used to examine chlorination products of drinking water, and this approach has been invaluable for isolating and characterizing many drinking water chemicals and for establishing the mutagenic activity of these materials and some of their fractions in bacterial systems (*8–15*). These considerations led to the selection of Fluka humic acid for the bioassays described in this report.

The chlorination products showed mutagenic activity in *S. typhimurium* tester strains TA 98 and TA 100.

Table 11. Summary of tumor incidences (benign and malignant) in bioassays of chlorinated humic acids.

	Mice with leukemia, %		Solid tumor, %		Mice with tumor, %	
Treatment	Females	Males	Females	Males	Females	Males
Chlorinated humic acids (HACl), 1:1	70	58	60	68	84	88
HACl, 1:0.3	64	34	52	72	88	86
HA	82	32	52	78	88	84
NaCl	60	40	54	70	80	86
No treatment	63	23	57	67	84	76
DBE	10	2	98	96	98	96

Table 12. Summary of significant tumor incidences in bioassay of chlorinated humic acids.[a]

Treatment	Tumor	
	Females	Males
HACl, 1:1	Lymph node, 5 hemangioma ($p < 0.005$)	None
	Ovaries/oviducts, 3 hemangioma ($p \cong 0.05$)	
	Harderian/lacrimal gland, 5 papillary adenoma, $p \cong 0.05$	
HACl, 1:0.3	Lymph node, 2 hemangioma ($p \cong 0.05$)	Prostate gland, 2 adenocarcinoma ($p < 0.05$)
	Brain, 2 hemangioma ($p < 0.05$)	Skeletal system, 2 *osteoma* ($p < 0.05$)
HA	Lymph node, 2 hemangioma ($p \cong 0.05$)	None

[a] Significance compared with untreated controls. It should be noted that none of the above tumor incidences was significantly different from the NaCl control groups.

In agreement with earlier observations (*19*), this mutagenic activity was found to be greater in the absence of an S9 liver microsomal activating system than in its presence. Thus, most of the chemicals responsible for this mutagenic activity are direct-acting mutagens. This observation, when combined with the observed alkylating activity of chlorinated humic acids, is important with regard to the possible carcinogenicity of chlorination products in rodent bioassays. We also examined the increase in alkylating activity and mutagenicity with increasing TOX levels. At the lower C:Cl ratio, 1:0.3, alkylating activity was not observed by the PNBP method, which we ascribe to inadequate sensitivity of this method. Mutagenic activity, as determined in this work, was also found to persist in chlorinated humic acids after fractionation by molecular weight and after freeze-drying (*23*).

For the purposes of discussion, the total tumor incidences in the chronic bioassay reported here are given in Table 11 as a percentage of animals tested with tumors (benign and malignant). The positive control, DBE, resulted in the expected high incidence of animals with tumors (*18*), which supports the contention that these animals are suitable for chronic bioassay of humic acids. The only notable observation regarding tumor incidences in the test groups compared to controls was an increased incidence of leukemia in males in the chlorinated humic acid 1:1 group, which was more than double the incidence in the no-treatment controls but not significantly different from that of the NaCl controls. No other pattern of tumorigenicity emerged from an examination of the tumor incidences presented in Table 11. A few marginally significant ($p \cong 0.05$) to significant ($p < 0.05$ to $p < 0.005$) incidences of solid tumors occurred, and these are listed in Table 12. Administration of chlorinated humic acid, 1:0.3, to male mice resulted in animals with adenocarcinoma of the prostate gland and two with osteoma. Significant incidences of tumors were not observed in males exposed to the higher level of chlorinated humic acid, 1:1. Hemangiomas of the lymph nodes were observed in females exposed to both levels of chlorinated humic acids, unchlorinated humic acid, and NaCl, and hence chlorination of humic acids does not account for this low incidence of hemangiomas in females.

A recently published report (*27*), described the use of a concentrate of mutagenic organics in drinking water for chronic bioassay in male and female Wistar rats. The incidence of tumors in these animals did not differ significantly from those in untreated control groups. The authors pointed out that the absence of tumorigenic response may be attributable to the low levels of mutagenic organics administered to the animals in drinking water.

In summary, chlorinated humic acids administered to both sexes of a widely used strain of mice in acceptable group sizes and at high dose levels resulted in only a few animals with significant numbers of tumors that were not related to the chlorination of humic acids.

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